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13. ABSTRACT (Maximum 200 Words)

Erythropoietin (Epo) is induced by hypoxia and stimulates the proliferation and inhibits the apoptosis of Epo receptor (EpoR) bearing erythroblasts in the bone marrow. Hypoxia in human cancer is associated with invasion, metastasis, resistance to therapy. We have recently shown hypoxia-stimulated expression of Epo and EpoR in human breast cancer cell lines, suggesting a role for Epo signaling in breast cancer biology. We hypothesized that hypoxia induces increased expression of Epo and EpoR in breast cancers, and the increased Epo signaling results in inhibition of tumor cell apoptosis. Our aim during the second year of the proposed study was to determine the spatial distribution and correlation of tissue hypoxia as determined by EF5 binding with those of HIF-1α, Epo, EpoR and apoptotic activity in vivo in MCF-7 human breast cancer xenografts. We determined the intra- and intertumoral heterogeneity of apoptotic activity in the tumors. We have shown that tumor regions showing hypoxia are associated with significantly increased apoptotic activity. These regions also show increased Epo expression, and somewhat increased expression of the anti-apoptotic proteins bcl-2 and bcl-X_L, consistent with their induction by Epo signaling. Our results suggest, that similar to our *in vitro* findings, tissue hypoxia in breast cancers induces increased apoptosis. At the same time, it also induces increased Epo expression in the tumors, likely leading to increased expression of anti-apoptotic proteins and protection of tumor cells from apoptotic death.

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INTRODUCTION

Erythropoietin (Epo), produced in the kidney, is induced by hypoxia and stimulates the proliferation and inhibits the apoptosis of Epo receptor (EpoR) bearing erythroblasts in the bone marrow. Epo also plays a role in the protection of neurons from hypoxic damage by inhibiting their apoptosis. Hypoxia in cancer is associated with invasion, metastasis, resistance to therapy and is thought to select for tumor cells with diminished apoptotic potential. Hypoxia has been shown to be present in human breast cancers. We have recently shown the basal and hypoxia-stimulated expression of erythropoietin (Epo) and Epo receptor (EpoR) in human breast cancer cell lines and breast carcinomas, suggesting a role for Epo signaling in the hypoxia-induced survival of human breast cancers. We hypothesized that hypoxia induces increased expression of Epo and EpoR in breast cancers, and that via mechanisms similar to those present in erythroid cells and neurons, the hypoxia induced increased Epo signaling results in inhibition of tumor cell apoptosis. Our aim during the first year of the proposed study was to determine the effect of hypoxia on Epo and EpoR expression and induction of apoptosis in MCF-7 and HCC38 breast cancer cells. Further, we examined the effect of exogenous Epo treatment on hypoxia-induced apoptosis in the tumor cells. Our aim during the second year of the proposed study was to determine the spatial distribution of Epo and EpoR expression *in vivo* in MCF-7 xenografts, and determine wether there is correlation between their expression and the distribution of hypoxia and apoptotic activity in the tumors.

BODY

Task 1. To determine the effect of hypoxia on Epo and EpoR expression and induction of apoptosis in MCF-7 and HCC38 breast cancer cells (months 1-8)

a. To establish the effect of decreasing oxygen concentrations (0.01% - 10% oxygen) on Epo and EpoR expression in MCF-7 and HCC38 cells determined by RT-PCR, Western blotting and ELISA.
b. To establish the effect of decreasing oxygen concentrations on apoptotic activity in MCF-7 and HCC38 cells by detection of DNA fragmentation
c. To examine the effect of blocking the effect of endogenously produced Epo on hypoxia induced apoptosis using neutralizing anti-Epo antibody and soluble EpoR (sEpoR)

We have previously shown that MCF-7 breast cancer cells produce Epo and EpoR and reduced oxygen concentrations (1.0% oxygen) stimulate the expression of both proteins [1]. Hypoxia was shown to induce p53-dependent apoptosis in various cell systems, including MCF-7 breast cancer cells [2, 3]. Since in erythroid cells and neurons Epo signaling inhibits p53-dependent apoptosis by the increase of expression of bcl-2 and

bcl-X_L [4-7], we hypothesized that hypoxia-induced increase in Epo and EpoR expression in breast cancer cells leads to increased Epo signaling and inhibits hypoxia-induced apoptosis via mechanism similar to those described in other cell systems [4, 6]. To test our hypothesis *in vitro*, MCF-7 and HCC38 human breast cancer cells were exposed to various levels of hypoxia (0% to 10.0% oxygen concentration) using a well-characterized, finely controlled chamber system [8], and examined the expression of Epo and EpoR mRNA and protein using quantitative real time PCR and western blotting. Our results are summarized in publication #1 (please see appendix).

To determine the effect of decreasing oxygen concentrations on apoptotic activity, cells were exposed to various oxygen concentrations and tested for apoptotic activity. The effect of hypoxia on the level of the expression of pro- and anti-apoptotic proteins was examined by quantitative real time PCR and western blotting. The results are summarized in publication #1 (please see appendix).

Task 2. To determine the effect of exogenous Epo treatment on hypoxia-induced apoptosis in MCF-7 and HCC38 cells and establish MCF-7 xenografts in Balb/c mice (months 9-15)

a. To examine the effect of exogenous Epo treatment on hypoxia-induced apoptosis by detection of DNA fragmentation
b. To examine the effect of exogenous Epo treatment on the expression of the pro-apoptotic protein bax and the anti-apoptotic proteins bcl-2 and bcl-X_L by RT-PCR and Western blotting
c. To establish MCF-7 xenografts in athymic Balb/c mice and determine the intra- and intertumoral heterogeneity of apototic activity in the tumors (Months 10-15)

To investigate whether exogenously added Epo inhibits hypoxia-induced apoptosis, cells were treated with recombinant human Epo (Epoetin-α, rHuEpo, Amgen) and exposed to hypoxia. To assess the effect of endogenous Epo produced by the cells on hypoxia-induced apoptosis, a neutralizing polyclonal rabbit anti-human Epo antibody (R&D Systems) and recombinant soluble EpoR (sEPOR, R&D Systems), that have been shown to inhibit the effects of Epo in other systems [9], were added to the cultures. Apoptotic activity induced by hypoxia was assessed by TUNEL staining. In addition to apoptotic activity, we also assessed cell survival in MCF-7 cells after hypoxia exposure using the clonogenic survival assay, as described [10]. Since Epo inhibits apoptosis in erythroid cells by increasing the expression of bcl-2 and bcl-X_L [4-7], we investigated whether Epo may inhibit hypoxia-induced apoptosis in breast cancer cells via similar mechanisms. Real-time quantitative PCR and western blot analysis were performed to determine the effect of rHuEpo treatment on the expression of bcl-2, bcl-X_L and bad. The results are summarized in publication #1 (please see appendix).

We have grown MCF-7 tumor xenografts in 20 athymic Balb/c mice, as described previously [11]. Briefly, tumors were implanted subcutaneously in the flank of 4-6 week old female mice. At the time of injection of $5x10^6$ MCF-7 cells suspended in Matrigel (10 mg/ml, Collaborative Research), a pellet of 17β-estradiol (0.72 mg/pellet, Innovative Research of America) was implanted beneath the back skin. When the tumor was approximately 5 mm in diameter (Fig. 1), the estrogen source was removed and a tamoxifen-containing pellet (5 mg released over 60 days with a blood level of 3-4 ng/ml, Innovative Research of America) was implanted. Two days after the implantation of the pellets, tumors were studied for the presence apoptotic activity. To assess the inter- and intratumoral heterogeneity of apoptotic activity, sections of the tumors were stained for apotosis using TUNEL stain (TUNEL enzyme and TUNEL label, Roche), according to manufacturer's recommendations. TUNEL stained sections were interpreted by counting a mimimum of 500 cells per case in 10 random areas of the tumors. The mean apoptotic activity in the tumors ranged from 1.2 to 4.3 (average 2.4) (Fig. 2), which is very similar to data reported in the literature [3, 12, 13]. In general, tumors showing larger areas of necrosis showed higher apoptotic activity. When the apoptotic activity in various areas within individual tumors were compared, significant variation in the regional distribution of apoptotic activity was found. Within individual tumors, apoptotic activity in various areas ranged from 0 to 16. Thus, the intratumor heterogeneity of apoptotic activity in the xenografts was significantly higher than the mean intertumoral heterogeneity. Based on these data, we used the twenty established xenografts for further studies.

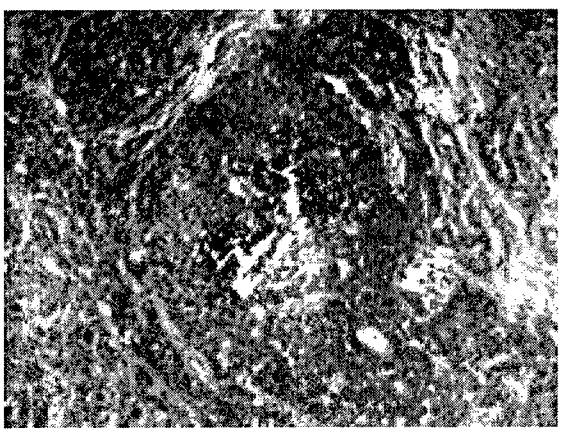

Fig. 1. Hematoxylin and eosin stained section of MCF-7 human breast cancer xenograft (original magnification 100X)

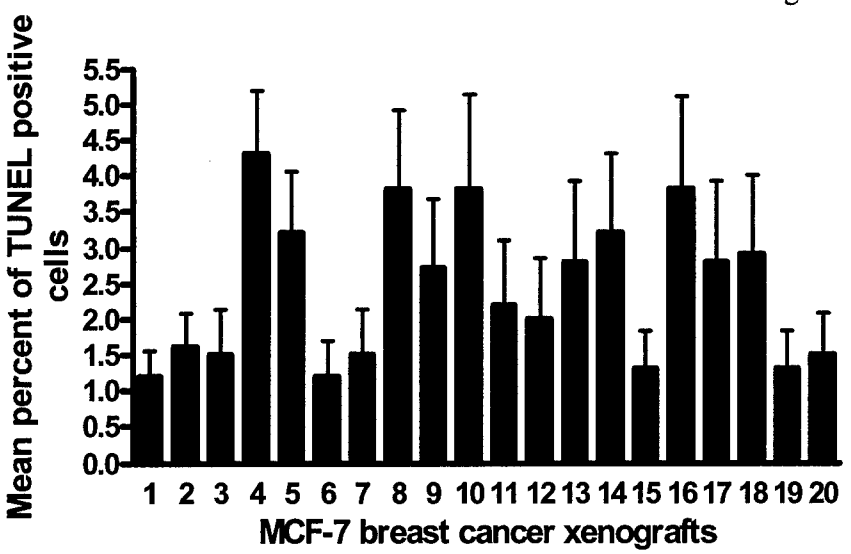

Fig. 2. Mean apoptotic activity in MCF-7 breast cancer xenografts determined by TUNEL staining.

Task 3. To determine the correlation of Epo and EpoR expression *in vivo* in MCF-7 xenografts with the distribution of tissue hypoxia and apoptotic activity (months 15-24)

b. To establish additional MCF-7 xenografts if necessary to achieve statistical power based on the results of the determination of intra- and intertumoral heterogeneity of apoptotic activity (Months 15-18).
c. To determine the distribution of tissue hypoxia in the xenografts using EF5 binding (Months 16-20)
d. To determine the expression of Epo, EpoR, HIF-1α, bax, bcl-2 and bcl-X_L and apoptotic activity in the tumor xenografts using immunohistochemistry and TUNEL staining, respectively (Months 180-24).

Tumor hypoxia in the xenografts was studied using immunohistochemical detection of nitroimidazole (EF5) binding as previously described [11, 14-16]. Briefly, tumor bearing mice were given two injections each of 10 mM EF5 prepared in 0.9% saline. The first injection was given i.v., and the second injection was given i.p. 15 minutes later. Three hours after EF5 administration, animals were sacrificed and the tumors were removed and frozen for sectioning. EF5 immunohistochemistry and image analysis to determine the extent and intensity of EF5 binding was performed as previously described [11, 14-16].

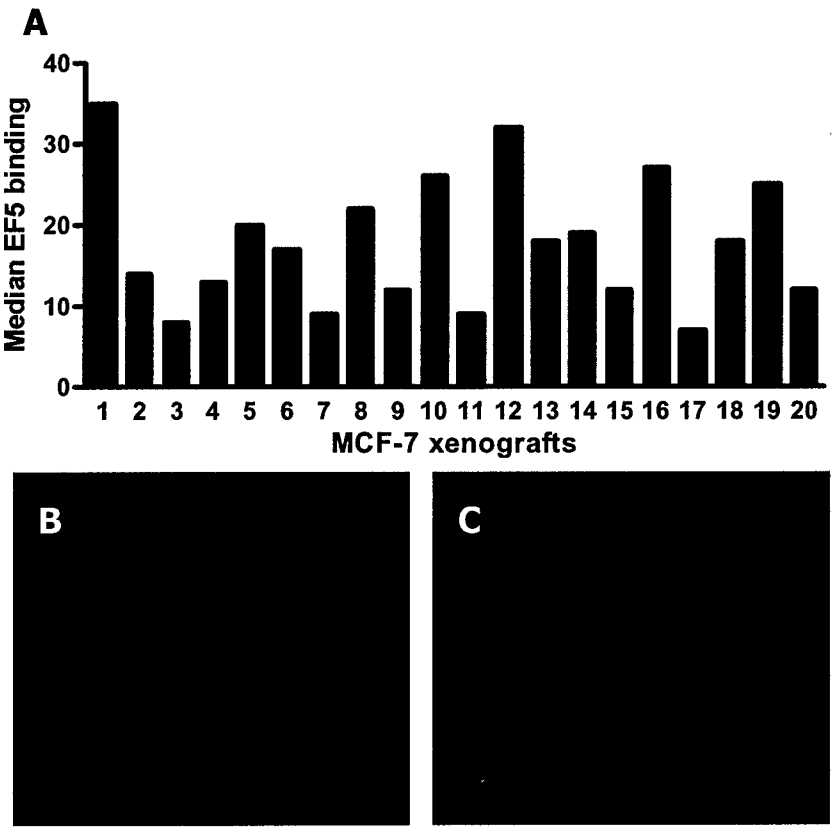

Fig.3. A. Median EF5 binding in 20 MCF-7 breast cancer xenografts. B and C: Patterns of EF5 binding in MCF-7 breast cancer xenografts. EF5 binding was detected by immunohistochemistry using a Cy3 labelled monoclonal antibody (red).

Although there was some intertumoral heterogeneity in EF5 binding, all tumors showed EF5 binding in at least 25% of the tumor area (Fig. 3). To

assess the spatial correlation between tumor hypoxia and apoptotic activity, sections of the tumors were double stained for apotosis using TUNEL stain and for EF5 binding (Fig. 4). TUNEL stained sections were interpreted by counting a mimimum of 500 cells per case in 3-3 areas of the tumors showing absent and present EF5 binding. Results were expressed as the percentage of cells showing TUNEL positivity. When areas showing EF5 binding (hypoxia) were compared to normoxic areas (not showing EF5 binding) within the same tumors, a significant difference in apoptotic activity was found: The apoptotic activity in hypoxic tumor areas (showing EF5 binding) was 7.4 ± 0.9 (mean ± SEM) compared to 1.2 ± 0.3 in normoxic areas lacking EF5 binding ($p < 0.0001$, unpaired t-test) (Fig. 4).

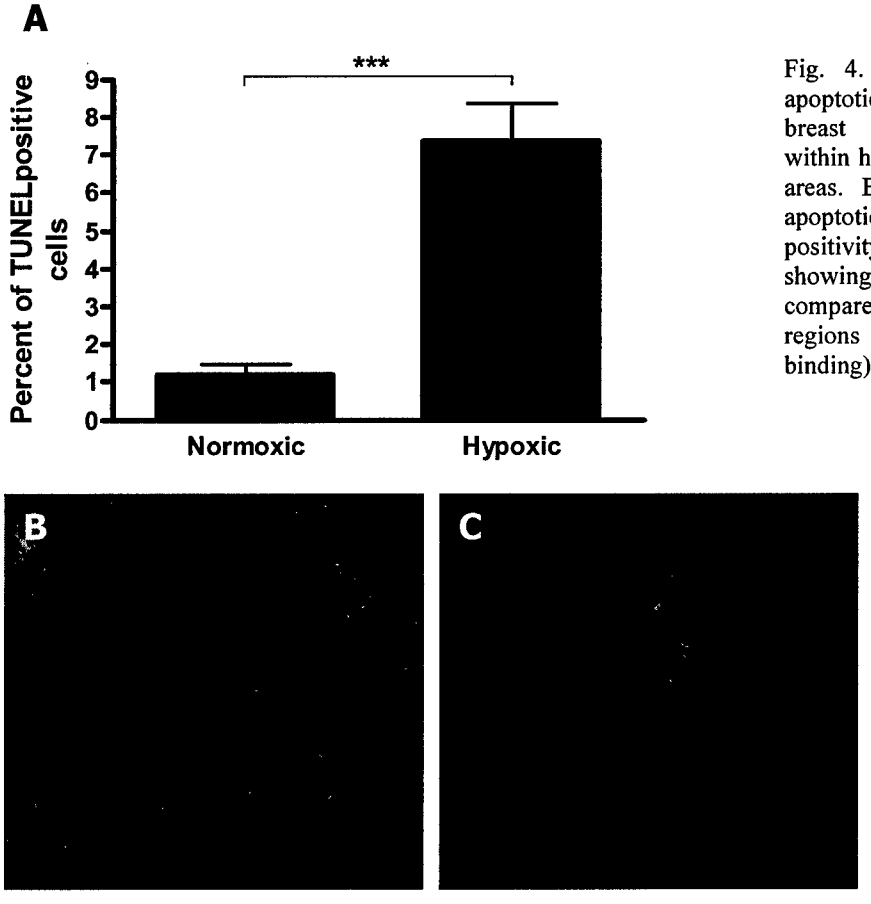

Fig. 4. A. Comparison of apoptotic activity in MCF-7 breast cancer xenografts within hypoxic and normoxic areas. B and C: Increased apoptotic activity (TUNEL positivity, green) in areas showing EF5 binding (red) compared to normoxic tumor regions (lack of EF5 binding).

Next, we used immunohistochemistry to determine the expression of Epo, EpoR, HIF-1α, bax, bcl-2 and bcl-X_L in the tumor xenografts. Immunohistochemical stains were performed as previously described [17, 18]. Representative images of the results are presented in Fig. 5. The expression of HIF-1α was heterogenous and concentrated aroud areas showing tumor cell necrosis. The expression of Epo was also heterogenous. While most tumor cells showed only weak staining, increased, strong immunoreac tivity was present in viable tumor cells around necrotic foci. Tumor regions surrounding areas of

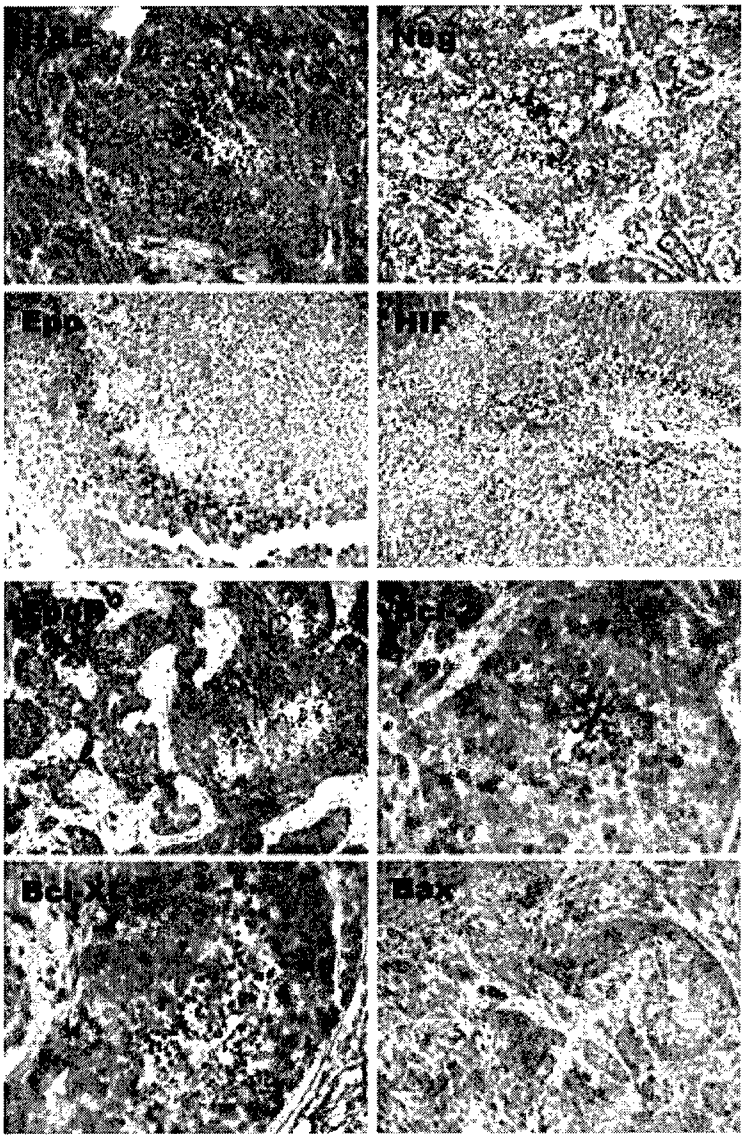

Fig. 5. Representative images of the results of immunohistochemical assays performed on MCF-7 breast cancer xenogarfts. H&E: hematoxylin and eosin stain; Neg: negative control.

necrosis are thought to be the most hypoxic parts of tumors. Indeed, such areas in the MCF-7 xenogarfts showed the highest levels of EF5 binding in our study. To examine the spatial distribution of HIF-1α and Epo in relation to tumor hypoxia, we performed double immunohistochemical stains for EF5 binding, HIF-1α and Epo expression (Fig. 6). Using these assays, we found that HIF-1α expression was localized to hypoxic tumor regions showing EF5 binding, consistent with the well known regulation of HIF-1α expression by tissue hypoxia. Similarly, high levels of Epo expression was also localized in hypoxic tumor regions showing EF5 binding and HIF-1α expression. This finding is consistent with the known regulation of Epo expression by HIF, and is also consistent with our prior *in vitro* results demonstrating increased Epo expression by MCF-7 cells under hypoxia. EpoR expression was strong and uniform in all tumors. Although we found EpoR expression to be increased by hypoxia in MCF-7 cells *in vitro*, we did not see increased EpoR expression in hypoxic regions of the tumor xenografts. The lack of increased EpoR

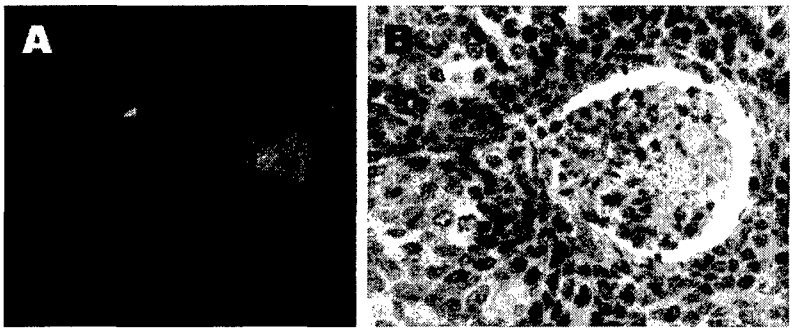

Fig. 6. A. Double immunohistochemical stain shows colocalization of HIF-1α expression (green) with tissue hypoxia (EF5 binding, red). B. Double immunohistochemical stain shows increased Epo expression (red) in regions showing HIF-1α expression (brown).

expression in such areas is likely due to the high basal level of EpoR expression in the tumors. The strong, uniform expression of EpoR in the tumors is similar to our findings in endometrial and cervical squamous cell carcinomas [17, 18]. The expression of bcl-2 and bcl-X_L was relatively similar in the tumors: both showed relatively homogenous staining with some increase near areas of necrosis. These results are in agreement with the results of our *in vitro* studies showing Epo induced increase in bcl-2 and bcl-X_L expression in MCF-7 cells. The expression of bax was also relatively uniform, and showed no appreciable change in perinecrotic tumor regions.

For the purposes of statistical analysis, immunostained sections were analysed semiquantitatively based on a four-tiered scale as described [17, 18]. At least 500 tumor cells were analyzed in 3-3 tumor regions showing increased EF5 binding, and/or Epo and HIF-1α expression. The percentage of weakly, moderately and strongly staining cells was determined, and a staining score was calculated as follows: Score (out of maximum of 300) = sum of 1 x percentage of weak, 2 x percentage of moderate and 3 x percentage of strong staining. The results are summarized in Fig. 7 and Table 1. HIF-1α and Epo expression was significantly higher in tumor regions showing EF5 binding compared to normoxic areas. No difference in EpoR expression was found between hypoxic and normoxic areas, likely due to the high basal level of EpoR expression. Both bcl-2 and bcl-X_L expression showed a trend towards increase in hypoxic areas, however the difference did not reach statistical significance. Similarly, no significant difference in bax expression was found between normoxic and hypoxic tumor regions.

Task 4. To determine the expression of Epo, EpoR and HIF-1α in human breast cancers and to correlate their expression with clinicopathological features and outcome (Months 19-36)

a. To select 250 patients with previously untreated, unilateral breast cancer for the study, review the available histological sections and select the appropriate tissue blocks for immunostaining (Months 19-24)

Dr. Solin, co-investigator of the proposed study, has provided the PI with a list of 300 patients treated with breast cancer at the University of Pennsylvania Medical Center. All slides have been obtained and are currently being reviewed by the PI to confirm the

diagnoses and to select suitable slides for the proposed study. We expect, that within two months all cases will be reviewed and slides selected for immunostaining.

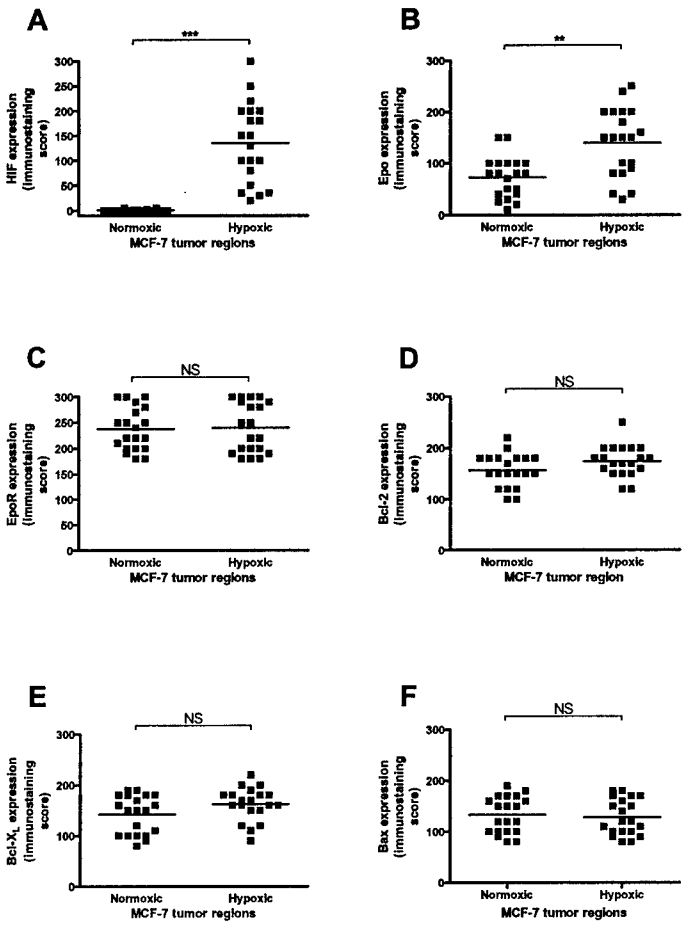

Fig. 7. Comparison of HIF-1α (A), Epo (B), EpoR (C), bcl-2 (D), bcl-X_L (E) and bax (F) expression in hypoxic and normoxic regions of MCF-7 breast cancer xenogarfts. ***, $p<0.0001$; **$p<0.01$; NS, not significant (Mann-Whitney test)

Table 1. Comparison of HIF-1α, Epo, EpoR, bcl-2, bcl-X_L and bax expression in hypoxic and normoxic regions of MCF-7 breast cancer xenografts

	Normoxic		Hypoxic		P*
	Median	Mean ± SEM	Median	Mean ± SEM	
HIF-1α	0	0.70 ± 0.35	140	135.50 ± 17.99	<0.0001
Epo	80	72.75 ± 8.87	150	139.50 ± 14.86	0.0019
EpoR	195	237.50 ± 9.26	200	240.00 ± 10.78	0.9784
Bcl-2	150	156.50 ± 7.27	175	174.50 ± 6.75	0.1067
Bcl-X_L	150	142.00 ± 8.45	165	162.50 ± 7.29	0.1070
Bax	135	133.00 ± 7.98	120	128.50 ± 7.86	0.7247

*Mann-Whitney test

KEY RESEARCH ACCOMPLISHMENTS

- We characterized the level of hypoxia (decreasing oxygen concentrations) necessary to induce expression of Epo and EpoR mRNA and protein *in vitro* in human breast cancer cells
- We characterized the level of hypoxia (decreasing oxygen concentrations) necessary to induce significant apoptotic activity *in vitro* in human breast cancer cells
- We characterized the changes in the level of mRNA and protein expression of pro- (bax) and anti-apoptotic (bcl-2 and bcl-X_L) proteins in response to hypoxia *in vitro* in human breast cancer cells
- We determined that exogenous rHuEpo decreased apoptotic activity and increased cell survival in human breast cancer cells exposed to extreme hypoxia *in vitro* (0% oxygen concentration)
- We determined that blocking the effect of endogenous Epo produced by the tumor cells enhances the apoptotic and cytotoxic effect of low oxygen concentrations *in vitro*
- We determined that treatment of human breast cancer cells *in vitro* with rHuEpo increased the level of bcl-2 and decreased the level of bax mRNA and protein expression, while it had no significant effect on bcl-X_L expression
- We established twenty MCF-7 breast cancer xenografts and determined the intra- and intertumoral heterogeneity of apoptotic activity in the tumors
- We determined *in vivo* the presence and distribution of tissue hypoxia by EF5 binding in MCF-7 breast cancer xenografts
- We determined that the expression of HIF-1α and increased expression of Epo *in vivo* in MCF-7 xenografts spatially correlates with the presence of tissue hypoxia as determined by EF5 binding
- We determined that *in vivo*, MCF-7 xenograft tumor regions showing hypoxia have significantly increased apoptotic activity compared to normoxic areas
- Expression of EpoR *in vivo* in MCF-7 breast cancer xenografts is uniformly strong and shows no significant correlation with tissue hypoxia, likely due to the high basal level of expression
- Expression of the anti-apoptotic proteins bcl-2 and bcl-X_L shows a trend toward increase in tumor regions showing high HIF-1α and Epo expression, consistent with the stimulation of their expression by Epo signaling; however these changes did not reach statistical significance
- No difference in the expression of the pro-apoptotic protein bax was found between tumor regions showing high or low HIF-1α and Epo expression

REPORTABLE OUTCOMES

- We have presented a poster at the 95th Annual Meeting of the American Association for Cancer Research, Orlando, Florida, March 27-31, 2004, entitled "Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast cancer cells" by Acs, G., Chen, M., Xu, X. and Koch, C.J

- We have published our results on our in vitro studies: Acs, G., Chen, M., Xu, X., Acs, P., Verma, A. and Koch, C.J.: Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells. Cancer Lett, 214: 243-251, 2004 (see Publication #1 in Appendix)
- We are currently preparing a manuscript summarizing the results of our *in vivo* studies for publication and plan to submit an abstract to be presented at the 96th Annual Meeting of the American Association for Cancer Research, to be held April 16-20, 2005 in Anaheim, CA.

CONCLUSIONS

We have shown that *in vitro*, decreased oxygen concentrations (hypoxia) induces Epo and EpoR expression and apoptosis in human breast breast cancer cells *in vitro*. However, the level of hypoxia to induce these responses is different: While Epo and EpoR expression is significantly increased at 1% oxygen concentration, significant apoptosis was only seen at extreme hypoxia (0% oxygen concentration). Hypoxia resulted in decreased expression of the anti-apoptotic protein bcl-X_L and increased expression of the pro-apoptotic protein bax. The resulting change in the ratio of these proteins may contribute to the increased apoptotic activity resulting from extreme hypoxia. Treatment of the tumor cells with exogenous rHuEpo resulted in increased expression of bcl-2 and decreased expression of bax, and reduced hypoxia-induced apoptotic activity in the tumor cells. Moreover, blocking the effect of endogenous Epo made by the tumor cells increased hypoxia induced apoptosis.

In vivo, we have shown that MCF-7 breast cancer xenografts show hypoxic areas as determined by EF5 binding, which are heterogeneously distributed within the tumor mass. We have shown that hypoxic tumor regions show increased expression of HIF-1α and Epo, which findings are in agreement with our *in vitro* results. Tumor regions showing hypoxia are associated with significantly increased apoptotic activity. However, these regions also show increased Epo expression, and somewhat increased expression of the anti-apoptotic proteins bcl-2 and bcl-X_L, consistent with their induction by Epo signaling. Since there were no hypoxic tumor regions that showed no Epo expression, we could not compare apoptotic activity induced by hypoxia in tumor regions with and without Epo expression. Our results suggest, that similar to our *in vitro* findings, tissue hypoxia in breast cancers induces increased apoptosis. At the same time, it also induces increased Epo expression in the tumors, likely leading to increased expression of anti-apoptotic proteins and protection of some tumor cells from apoptotic death. Future studies are planned to examine the effect of blocking hypoxia-induced Epo signaling in tumor xenografts by RNA interference, to examine whether such tumors are more susceptible to hypoxia induced cell death.

Our studies provide novel insight into the role of autocrine/paracrine Epo signaling in the hypoxic adaptation of breast cancers. We provided evidence for a novel mechanism whereby hypoxia not only selects for aggressive tumor phenotypes, but itself induces a mechanism in breast cancer cells to promote their hypoxic survival. These adaptive changes in the tumor cells may play an important role in tumor progression. It is

also well known that tumor hypoxia is associated with resistance to radiation and chemotherapy, treatment modalities that induce apoptosis in the tumor cells. Our studies suggest that hypoxia-induced increase in Epo signaling and the resulting inhibition of apoptosis may provide a mechanism for the resistance of hypoxic tumor cells to radiation- and chemotherapy. This hypothesis is also supported by our recent findings that increased Epo expression in human endometrial carcinomas is associated with adverse outcome and appears to be an independent prognostic/predictive factor [17]. The widespread use of rHuEpo for treatment of chemotherapy-related anemia in breast cancer patients adds immediate clinical significance to our studies. Very recently, a multicenter, prospective, randomised study on the effect on rHuEpo administration to breast cancer patients undergoing chemotherapy was terminated unexpectedly because of the observed higher mortality of patients treated with rHuEpo [19]. The observed difference in deaths was mainly due to an increase in incidence of disease progression in the rHuEpo treated group. Our results suggest that biologically active Epo signaling in the tumor cells might contribute to such adverse effects of rHuEpo treatment.

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Autocrine erythropoietin signaling inhibits hypoxia-induced apoptosis in human breast carcinoma cells

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Abstract

Disordered perfusion and the resulting hypoxia are important features conferring tumor heterogeneity, which may contribute to relapse. Hypoxic tumor cells have been associated with resistance both to radiation and to cytotoxic drugs. Hypoxia may also serve as a selection pressure in tumors by promoting apoptosis of some cells and expanding variants with decreased apoptotic potential, and thus play a role in the development of a more aggressive phenotype. Erythropoietin (Epo), induced by hypoxia, controls erythropoiesis and plays a role in protection of neurons from hypoxic damage. We have recently demonstrated hypoxia-stimulated expression of Epo and Epo receptor (EpoR) in human breast and cervix cancers, suggesting a role for autocrine Epo signaling in the hypoxic adaptations of carcinomas. In the current study we provide evidence that increased autocrine Epo signaling induced by moderate levels of hypoxia inhibits hypoxia-induced apoptosis and promotes survival in MCF-7 human breast cancer cells. The anti-apoptotic effect of Epo correlates with upregulation of bcl-2 and bcl-X_L, suggesting a mechanism similar to those described in hematopoietic cells. The resulting decreased apoptotic potential of hypoxic tumor cells may contribute to increased aggressiveness and therapy resistance of breast cancers.

Keywords: Erythropoietin; Erythropoietin receptor; Hypoxia; Breast carcinoma; MCF-7 cells

1. Introduction

Disordered perfusion and unregulated growth in cancer results in low regional oxygen supply within solid tumors [1,2]. The low level of oxygen in human breast and other cancers has been documented with micro-electrodes [3–5] and is considered to be a therapeutic problem, as it makes solid tumors resistant to radiation and chemotherapy [5]. Several studies have shown that intratumoral hypoxia is positively correlated with invasiveness, metastasis and overall adverse clinical outcome [6–8]. Despite the clinical

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importance of these observations, the molecular basis of invasiveness and metastasis is not well understood.

Cells adapt to hypoxic stress via cell-specific protective mechanisms such as cell cycle arrest, reduced energy dependent protein synthesis and selective gene induction [9–11]. An immediate consequence of hypoxia is the stabilization of hypoxia-inducible factor-1 (HIF-1), which in turn stimulates transcription of a number of genes important for tumor cell survival and tumor progression [12]. HIF-1 expression is also highly correlated with malignant progression of cancer. However, despite the induction of these protective mechanisms, hypoxia also irreversibly damages and kills cells [13]. In fact, diffusion limited, or ischemic necrosis is a common feature of solid tumors. Although cell death due to hypoxia can be associated with necrosis [14], recent observations suggested the possibility of hypoxia-induced apoptosis [2,13,15]. The level of hypoxia required to cause apoptosis has not been well characterized, but severe hypoxia (less than 0.05% oxygen in gas phase for extended times) has been reported to cause apoptosis in vitro [2,15]. Wild-type p53 seems to play a key role in the regulation of hypoxia-induced apoptosis [2,13] and genetic alterations, such as the loss of p53 or overexpression of bcl-2, can substantially reduce hypoxia-induced apoptosis [2,16]. Hypoxia itself appears to be capable of promoting genetic instability [17,18]. Tumor hypoxia may thus serve as a physiological selection pressure in tumors by promoting apoptosis of some cells and expanding variants that have lost their apoptotic potential [2].

Erythropoietin (Epo), a glycoprotein hormone produced in the kidney, is considered to be a specific stimulator of erythropoiesis [19–21]. Epo gene expression is primarily modulated by tissue hypoxia mediated by HIF-1 [19,22,23]. In erythroid cells binding of Epo to its receptor (EpoR) activates multiple signaling pathways (reviewed in Ref. [24]) resulting in the stimulation of proliferation and differentiation, increased expression of the anti-apoptotic proteins bcl-2 and bcl-X_L [25,26], and inhibition of apoptosis [23,24,27–30]. Both Epo and EpoR have recently been found to be expressed by a variety of other cell types, including endothelial cells [31], neurons [32], trophoblast cells [33] and mammary epithelial cells [34–36]. This suggests a wider biological role for Epo signaling unrelated to erythropoiesis [37–39]. As in hematopoietic cells, Epo has been shown to be a potent inhibitor of neuronal apoptosis induced by ischemia and hypoxia in vitro and in vivo [40]. Epo also inhibits interleukin-1β, sodium nitroprusside and lipopolysaccharide-induced apoptosis in endothelial cells [41,42], and cytokine-induced apoptosis in pancreatic islet cells [43].

We have recently reported that cultured human breast and cervix cancer cell lines and carcinomas express high levels of Epo and EpoR mRNA and protein [35,44]. Exposure of the tumor cells to recombinant human Epo (rHuEpo) stimulated tyrosine phosphorylation, DNA synthesis and proliferation, indicating that Epo signaling is biologically active. In the current study we examined the hypothesis that in addition to the induction of apoptosis at very low O_2 concentrations, moderate hypoxia also induces increased expression of Epo and EpoR in breast cancer cells and the resulting increased autocrine/paracrine Epo signaling contributes to the hypoxic survival of cancer cells [35,44].

2. Materials and methods

2.1. Cell cultures and hypoxic treatments

The MCF-7 human breast carcinoma cell line, expressing wild type p53, was obtained from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were grown in DMEM (GIBCO Invitrogen Co., Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, ATCC), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml). For hypoxia treatment experiments cells were plated at a density of 1×10^6 cells per dish in 60 mm Permanox dishes (Nunc, Sewell, NJ) and cultured for 5 days. Twenty-four hours prior to hypoxia or rHuEpo treatments cells were switched to serum free medium. Hypoxia treatment of cells was performed using a well characterized, finely controlled chamber system as previously described [45] for the times indicated. The O_2 concentration in the chambers was assayed at the end of the incubation period using an oxygen sensitive electrode [45].

2.2. Quantitative real time PCR assay

RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). For RT-PCR 1 μg of total RNA per sample was reverse transcribed to cDNA using the SuperScript First-Strand Sysnthesis System (Invitrogen, Carlsbad, CA). Quantitative real time PCR was performed using the iCycler Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA). Amplification of specific PCR products was performed in a total reaction volume of 25 μl containing 5 μl cDNA template, sense and antisense primers, dual labeled fluorigenic internal probe and 1X IQ Supermix reagents (Bio-Rad Laboratories). Dual labeled nonextendable probes labeled with hexachlorofluorescein (HEX) at the 5′ end and with Black Hole Quencher™-1 (BHQ) at the 3′ end were used for detection of Epo, EpoR, bcl-2, bcl-X_L and bax. For the endogenous control 18S ribosomal RNA the probe was labeled with 6-carboxyfluorescein (6FAM) at the 5′ end and with BHQ at the 3′ end. Primers and probes for *Epo* (sense, 5′-CTGGAAGAGGATGGAGGTCGG-3′; antisense, 5′-GCTGGGAAGAGTTGACCAACAG-3′; probe, 5′-HEX-CCGCAGGACAGCTTCCGACAGCAG-BHQ-3′), *EpoR* (sense, 5′-CCTGACGCTCTCCCTCATCC-3′; antisense, 5′-GCCTTCAAACTCGCTCTCTGG-3′; probe, 5′-HEX-TCCTGGTGCTGCTGACCGTGCTCG-BHQ-3′), *bcl-2* (sense, 5′-GTGTGTGGAGAGCGTCAACC-3′; antisense, 5′-TCAGAGACAGCCAGGAGAAATCA-3′; probe, 5′-HEX-TCGCCCCTGGTGGACAACATCGCC-BHQ-3′), *bcl-X_L* (sense, 5′-CCACTTACCTGAATGACCACCTAG-3′; antisense, 5′-CAGCGGTTGAAGCGTTCCTG-3′; probe, 5′-HEX-CCCTTTCGGCTCTCGGCTGCTGC-BHQ-3′), *bax* (sense, 5′-AACTGGACAGTAACATGGAGCTG-3′; antisense, 5′-CTGGCAAAGTAGAAAAGGGCGA-3′; probe, 5′-HEX-TGATTGCCGCCGTGGACACAGACT-BHQ-3′) and *18S RNA* (sense, 5′-CGGAGGTTCGAAGACGATCAGATA-3′; antisense, 5′-TTGGTTTCCCGGAAGCTGCC-3′; probe, 5′-6FAM-TGGGAATAACGCCGCCGCATCGCC-BHQ-3′) were designed using the Beacon Designer software (version 2.1, Premier Biosoft International, Palo Alto, CA). PCR primers were designed to span an intron when possible to avoid amplification of genomic DNA. Amplifications were performed at 95 °C for 3 min and for 40 cycles of 30 s at 95 °C and 30 s at 60 °C. Change in gene expression relative to the 18S RNA endogenous control was determined by the following formula: Fold change $= 2^{-\Delta}(\Delta CT)$, where $\Delta CT = CT_{target} - CT_{18S\ RNA}$, and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$ (CT, threshold cycle).

2.3. Western blotting

Whole cell lysates were normalized for protein. Fifty micrograms of proteins from each sample were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Proteins were detected using rabbit polyclonal antibodies to Epo (H-162, 1:500 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), EpoR (C-20, 1:1500 dilution, Santa Cruz), bax (1:1000 dilution, Cell Signaling Technology, Beverly, MA), bcl-X_L (1:1000, Cell Signaling Technology), and mouse monoclonal antibody to bcl-2 (clone 124, 1:200 dilution, DAKO Cytomation, Carpinteria, CA). As a loading control, a mouse monoclonal antibody to β-actin (clone AC-74, 1:10,000 dilution, Sigma, St Louis, MO) was used. Membranes were incubated with the primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Bio-Rad Laboratories, Hercules, CA). Immunoreactive bands were visualized using chemiluminescence (ECL Plus Western Blotting Detection System, Amersham Biosciences, Little Chalfont, England).

2.4. TUNEL assay for apoptosis

Cells were plated as above and cultured for 5 days. Twenty-four hours before hypoxia treatment cells were switched to serum free medium. Cells were pretreated for 2 h with human recombinant Epo (rHuEpo, Epogen®, Epoetin alfa, Amgen, Thousand Oaks, CA), human recombinant soluble EpoR (sEpoR, R&D Systems, Minneapolis, MN) or blocking anti-Epo antibody (rabbit polyclonal, R&D Systems) and exposed to various concentrations of oxygen for 24 h. Both adherent and floating cells were harvested for the apoptosis assays. Cells were washed in PBS, fixed in 1% paraformaldehyde for 10 min at 4 °C and permeabilized with 0.1% Triton-X100. Apoptosis was assayed by detection of DNA fragmentation by terminal deoxynucleotidyl transferase

(TdT) mediated dUTP-biotin nick end labeling (TUNEL) using TUNEL enzyme and TUNEL Label (Roche, Indianapolis, IN) according to manufacturer's recommendations. Flow cytometric analyses were performed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). In each experiment at least 10,000 cells were analyzed.

2.5. Clonogenic survival assay

Cell survival was assessed by means of the cells' abilities to form visible colonies. After hypoxia exposure for 24 h, cells were harvested by trypsinization. Cells were counted using both a particle counter and hemocytometer. Two hundred and fifty cells were plated in 10 cm culture dishes in replicates of five and incubated for 14 days with change of medium every 5 days. Cells were then fixed in methanol and stained with methylene blue. Colonies composed of at least 50 cells were counted. The surviving fraction is expressed as the number of colonies relative to the number of colonies in the untreated control.

3. Results

Exposure of MCF-7 cells to increasingly severe hypoxia (decreasing O_2) resulted in a dose dependent increase in Epo mRNA levels (Fig. 1A). Moderate levels of hypoxia (1% O_2) induced a 2.1 ± 0.4-fold increase in Epo mRNA expression, while maximal increase (7.5 ± 0.9-fold) was seen at severe hypoxia ($<0.005\%$ O_2). Similarly, increasing hypoxia resulted in an increase in EpoR mRNA expression, with 1.7 ± 0.4-fold increase at 1% O_2, and a maximal increase of 2.3 ± 0.5-fold seen at 0.1% O_2 concentration. In contrast to Epo, EpoR mRNA expression decreased to basal level at $<0.005\%$ O_2 concentration. Western blot analysis showed a change in the expression levels of Epo and EpoR protein very similar to that seen on the mRNA level (Fig. 1B).

The basal apoptotic activity in MCF-7 cells cultured under aerobic conditions was $1.8 \pm 0.2\%$ as measured by TUNEL staining (Fig. 2A). Moderate levels of hypoxia did not induce a significant increase in apoptotic activity: after exposure to 1 and 0.3% O_2 concentrations for 24 h the percentage of TUNEL positive cells was 3.7 ± 1.9 and $4.1 \pm 2.0\%$, respectively. In contrast, severe hypoxia ($<0.005\%$ O_2 for 24 h) induced a significant increase in the number of TUNEL positive cells ($44.2 \pm 9.3\%$). We did not observe any significant apoptotic activity after exposure to hypoxic conditions for 2–12 h (not shown). We next examined the expression levels of bcl-2, bcl-X_L and bax mRNA and protein. There was no change in the level of bcl-2 expression in MCF-7 cells exposed to hypoxia (Fig. 2B and C). In contrast, a dose dependent decrease in the expression of bcl-X_L mRNA and protein was found (Fig. 2B and C): maximal decrease in bcl-X_L mRNA expression (relative copy number: 0.31 ± 0.11) was seen at severe hypoxia ($<0.005\%$ O_2). Moderate levels of hypoxia (3 and 1% O_2) induced a moderate increase in bax mRNA and protein expression (Fig. 2B and C).

To examine the effect of exogenous Epo treatment on hypoxia-induced apoptotic activity, MCF-7 cells were pretreated with rHuEpo (200 U/ml) for 2 h before exposure to hypoxia. In cells exposed to severe

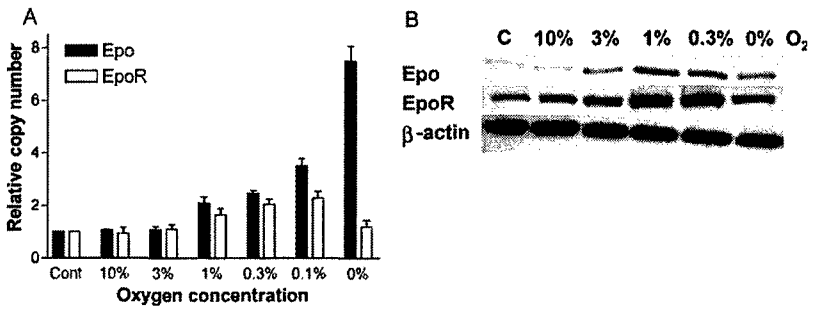

Fig. 1. (A) Effect of decreasing oxygen concentrations on the expression of erythropoietin (Epo) and Epo receptor (EpoR) mRNA in MCF-7 cells. Data represent mean ± SEM of three independent experiments. (B) Effect of decreasing oxygen concentrations on Epo and EpoR protein expression in MCF-7 cells; blots were also stained for β-actin as a loading control.

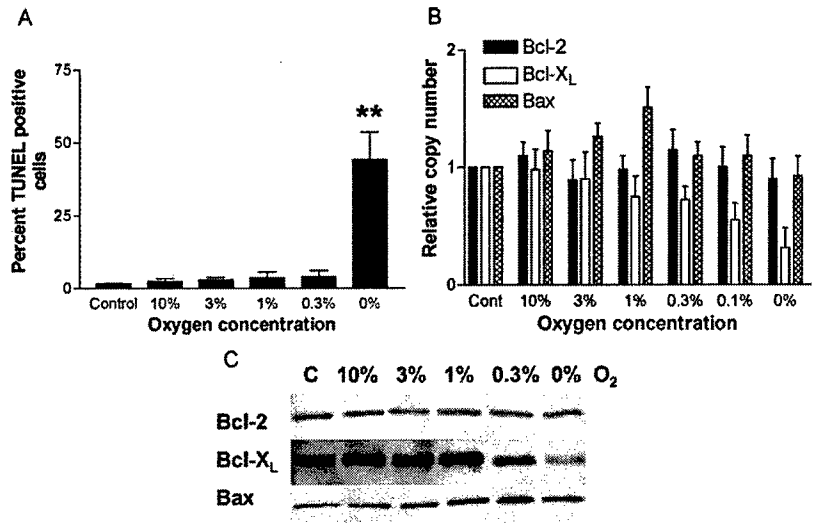

Fig. 2. (A) Effect of decreasing oxygen concentrations on apoptotic activity in MCF-7 cells measured by TUNEL staining. Data represent mean ± SEM of three independent experiments. (**, $P<0.01$, t-test) (B) Effect of decreasing oxygen concentrations on the expression of bcl-2, bcl-X_L and bax mRNA in MCF-7 cells. Data represent mean ± SEM of three independent experiments. (C) Effect of decreasing oxygen concentrations on the expression of bcl-2, bcl-X_L and bax protein in MCF-7 cells.

hypoxia ($<0.005\%$ O_2), rHuEpo treatment resulted in decreased hypoxia-induced apoptotic activity (34.5 ± 4.9% TUNEL positive cells compared to 43.2 ± 8.4% in untreated cells) (Fig. 3A). Exogenous rHuEpo had no significant effect on apoptotic activity in cells exposed to moderate levels of hypoxia. Since MCF-7 cells express Epo and hypoxia stimulates their Epo expression, we examined whether blocking the effect of endogenous Epo using sEpoR or anti-Epo blocking antibody has an effect on hypoxia-induced apoptosis in the cells. Pretreatment of the cells with either sEpoR (100 ng/ml) or anti-Epo antibody (100 μg/ml) for 2 h before hypoxia treatment resulted in increased apoptotic activity at severe hypoxia ($<0.005\%$ O_2) (Fig. 3A). At moderate hypoxia (1% O_2), the ratio of TUNEL positive cells also showed a trend toward increase that did not reach statistical significance.

To examine the effect of exogenous Epo, and blocking of endogenous Epo on the survival of cells exposed to hypoxia, we used the clonogenic survival assay. In MCF-7 cells exposed to moderate levels of hypoxia (1% O_2) exogenous rHuEpo increased, while blocking the effect of endogenous Epo by sEpoR or anti-Epo antibody decreased the ratio of surviving, colony forming cells (Fig. 3B). Exogenous rHuEpo also increased survival in cells exposed to severe hypoxia ($<0.005\%$ O_2), while blocking endogenous Epo had no significant effect on cell survival under these conditions.

Since in erythroid cells and neurons Epo exerts its anti-apoptotic effect by increasing the expression of the anti-apoptotic proteins bcl-2 and bcl-X_L [25,26, 46], we examined whether exogenous Epo treatment had an effect on the expression level of these proteins in MCF-7 cells. As shown in Fig. 3C and D, rHuEpo treatment induced increased expression of both bcl-2 and bcl-X_L mRNA and protein, with maximal effect seen at 100 U/ml rHuEpo concentration. In the case of the pro-apoptotic protein bax, a slight decrease in mRNA and protein expression levels was seen.

4. Discussion

We have provided evidence that increased autocrine Epo signaling induced by moderate levels of hypoxia inhibits hypoxia-induced apoptosis and promotes survival in MCF-7 human breast cancer cells. The anti-apoptotic effect of Epo correlated with upregulation of bcl-2 and bcl-X_L, thus its mechanisms appear to be similar to those described in hematopoietic cells [26]. The potency of Epo for inhibiting hypoxia-induced apoptosis and promoting survival was comparable to those described for Epo for

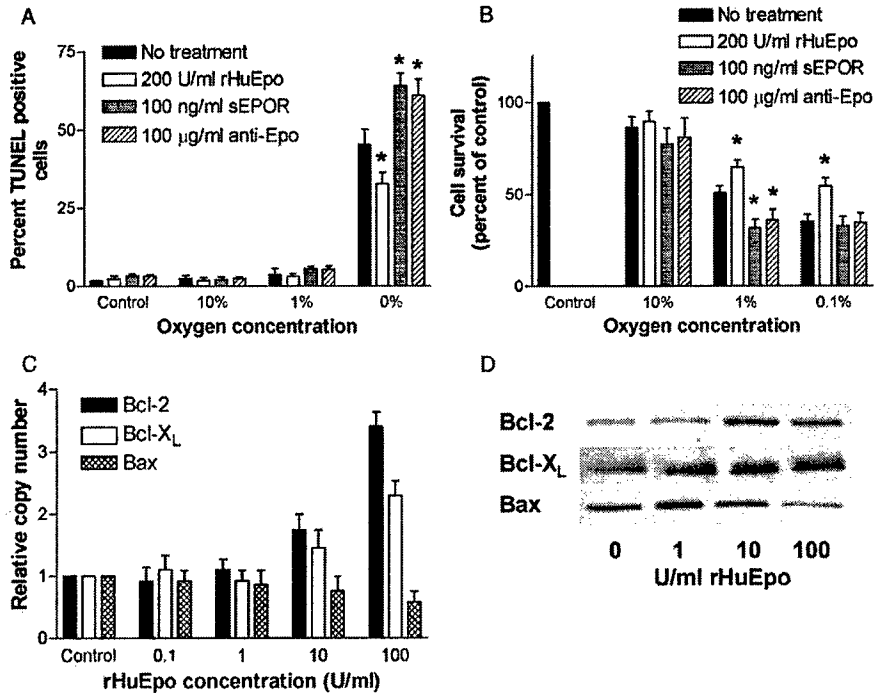

Fig. 3. (A) Effect of exogenous human recombinant erythropoietin (rHuEpo), and blocking the effect of endogenous erythropoietin (Epo) with soluble Epo receptor (sEpoR) or anti-Epo antibody on hypoxia-induced apoptosis in MCF-7 cells. Apoptosis was assessed by TUNEL staining. Data represent mean ± SEM of three independent experiments. (*, $P < 0.05$, *t*-test) (B) Effect of exogenous rHuEpo, and blocking the effect of endogenous Epo with soluble Epo receptor (sEpoR) or anti-Epo antibody on clonogenic cell survival after exposure to hypoxia in MCF-7 cells. Data represent mean ± SEM of three independent experiments. (*, $P < 0.05$, *t*-test) (C) Effect of exogenous rHuEpo on the expression of bcl-2, bcl-X_L and bax mRNA in MCF-7 cells. Data represent mean ± SEM of three independent experiments. (D) Effect of exogenous rHuEpo on the expression of bcl-2, bcl-X_L and bax protein in MCF-7 cells.

inhibition of lipopolysacharide-induced apoptosis in endothelial cells [41]. Similarly, the concentration of Epo (200 U/ml) for maximal stimulation of bcl-2 and bcl-X_L mRNA and protein expression was similar to that reported in human neuroblastoma and Ewing's sarcoma cells [47].

Evasion of apoptosis, through the activation of survival pathways, plays an important role in the development of cancer and is now widely regarded as a hallmark of malignancy [48]. Growth factors can inhibit apoptosis through increased expression of prosurvival genes and through posttranslational modification and inactivation of proapototic proteins. Many tumors are capable of synthesis of growth factors to which they are responsive, creating autocrine signaling mechanisms to stimulate their growth and decrease apoptosis [48]. We have recently shown that breast cancer cells express both Epo and its receptor, suggesting a role for autocrine Epo signaling in their biology [35].

The frequent ability to achieve remission followed by relapse implies that within an individual patient, tumor cells are not homogenous in their treatment sensitivities [1]. Disordered tumor cell perfusion and the resulting hypoxia have been suggested to be particularly important features conferring tumor heterogeneity, which may contribute to relapse [1]. Hypoxic cells have long been associated with resistance both to radiation and to cytotoxic drugs [49–51]. Experimental data suggest that one of the major modes of action of chemotherapeutic drugs and irradiation may be via activation of apoptosis [52–54]. In addition, overexpression of apoptosis-promoting or apoptosis-suppressing genes can modify radiation-induced cell

survival and radiosensitivity [54]. We have previously shown that Epo can inhibit chemotheraputic drug-induced apoptosis and cytotoxicy [44]. Our current results suggest that the increased Epo signaling induced by tumor hypoxia can play a significant role in the therapy resistance of hypoxic tumors. While in most studies hypoxia has been considered a dichotomous variable, i.e. tumors and tumor cells are considered either normoxic or hypoxic, experimental data document the existence and significance of a continuum of oxygen tensions in tumor cells [55]. Recent evidence also suggests that the percentage of moderately, not severely hypoxic cells determine response to therapy [56]. Our results that increased autocrine Epo signaling occurs at moderate levels of hypoxia and promotes tumor cell survival also support the biologic importance of moderately hypoxic tumor cells.

In addition to its role in treatment resistance, recent studies indicate that hypoxia may also be involved in the development of a more aggressive phenotype per se, and may contribute to metastasis [7,8]. Altering components of the apoptotic machinery can dramatically affect the dynamics of tumor progression. A critical role of p53-induced apoptosis for p53-mediated tumor suppression in vivo has been demonstrated [57,58]. Although hypoxia-induced cell death is often considered to be necrosis [14], recent observations and our current results suggest that depending on the degree and duration of hypoxia, either apoptosis or necrosis may result [13,15,59]. Hypoxia-induced apoptosis is thought to be p53-dependent [2,13]. It has been observed that p53-mediated apoptosis can be prevented by the anti--apoptotic proteins bcl-2 and bcl-X_L [2,60]. In fact Epo normally exerts its erythropoietic actions by blocking the p53-mediated default apoptosis of erythroblasts via upregulation of bcl-2 and bcl-X_L [26]. Inactivation of p53-dependent apoptosis, such as apoptosis induced by hypoxia, can provide a selective advantage at any point during tumor progression [61]. It was suggested that tumor hypoxia selects for cells with diminished apoptotic potential [2], however the mechanism of this selection is not known. Our results suggest that hypoxia-induced increased Epo signaling in cancer cells can play a role in this phenomenon by contributing to inactivation of apoptosis. This mechanism may play a role in tumor progression. This hypothesis is further supported by our recent observation that increased Epo expression in endometrial carcinomas appears to be an independent prognostic factor [62].

In summary, our results suggest that moderate levels of hypoxia stimulates autocrine Epo signaling in human breast cancer cells and, via mechanisms similar to those present in erythroid cells, contributes to decreased apoptosis. The decreased apoptotic potential of hypoxic tumor cells could contribute to increased aggressiveness and therapy resistance of breast cancers, and lead to adverse outcome.

5. Summary

Tumor hypoxia is associated with treatment resistance and promotes an aggressive phenotype with decreased apoptotic potential. We provide evidence that increased autocrine Epo signaling induced by moderate levels of hypoxia inhibits hypoxia-induced apoptosis and promotes survival in MCF-7 human breast cancer cells. The anti-apoptotic effect of Epo correlates with upregulation of bcl-2 and bcl-X_L, suggesting a mechanism similar to those described in hematopoietic cells.

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